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Glucocorticoid-regulated Localization of Cell Surface Glycoproteins in Rat Hepatoma Cells Is Mediated within the Golgi Complex

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Abstract. Glucocorticoid hormones regulate the post-translational maturation and sorting of cell surface and extracellular mouse mammary tumor virus (MMTV) glycoproteins in M1.54 cells, a stably infected rat hepatoma cell line. Exposure to monensin significantly reduced the proteolytic maturation and externalization of viral glycoproteins resulting in a stable cellular accumulation of a single 70,000-M_r glycosylated polyprotein (designated gp70). Cell surface- and intracellular-specific immunoprecipitations of monensin-treated cells revealed that gp70 can be localized to the cell surface only in the presence of 1 μM dexamethasone, while in uninduced cells gp70 is irreversibly sequestered in an intracellular compartment. Analysis of oligosaccharide processing kinetics demonstrated that gp70 acquired resistance to endoglycosidase H with a half-time of 65 min in the presence or absence of hormone. In contrast, gp70 was inefficiently galactosylated after a 60-min lag in uninduced cells while rapidly acquiring this carbohydrate modification in the presence of dexamethasone. Furthermore, in the absence or presence of monensin, MMTV glycoproteins failed to be galactosylated in hormone-induced CR4 cells, a complement-selected sorting variant defective in the glucocorticoid-regulated compartmentalization of viral glycoproteins to the cell surface. Since dexamethasone had no apparent global effects on organelle morphology or production of total cell surface–galactosylated species, we conclude that glucocorticoids induce the localization of cell surface MMTV glycoproteins by regulating a highly selective step within the Golgi apparatus after the acquisition of endoglycosidase H–resistant oligosaccharide side chains but before or at the site of galactose attachment.

Newly synthesized glycoproteins destined for the plasma membrane or extracellular environment undergo a succession of compartment-specific processing reactions during transit through the cell (6, 8, 17, 30, 31, 45, 52, 53, 62, 64). For example, different Golgi cisternae contain specific glycosyltransferases and glycosidases which sequentially process the oligosaccharide portions of glycoprotein substrates (6, 8, 24, 31, 48). In addition, the polypeptide backbone may be proteolytically cleaved at specific loci (52, 64) or, for some integral membrane species, selectively modified by acylation in the *cis*-Golgi (7, 37, 43, 56). The intracellular transport and processing of many cell surface glycoproteins appears to proceed as part of the constitutive bulk flow movement of vesicle-compartmentalized proteins (17). However, several studies have suggested that during the sequential vesicular budding and fusion processes between various membrane organelles, constitutively transported species interact with specific recognition or carrier proteins which either retain polypeptide substrates in a given compartment or redirect them to other routes or cellular locations (3, 14–16, 52). For example, β_2-microglobulin binds and selectively mediates the transport of histocompatibility leukocyte antigen to the cell surface (32, 44) while a heavy chain–binding protein appears to regulate the movement of immunoglobulin heavy chains between the rough endoplasmic reticulum (RER)[1] and Golgi region (23). Carrier-mediated pathways have also been suggested to explain the differential intracellular transport of two classes of microvillus membrane hydrolases in human intestinal epithelial cells (22) and the selective localization of proteins to either the apical or basolateral membranes of polarized cells (38, 41, 46, 61). Moreover, cell surface proteins within the same cell can undergo distinct processing reactions or display differential transport kinetics between certain organelles (14, 52, 63). Thus, the precise control of entry into and through specific

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1. *Abbreviations used in this paper*: MMTV, mouse mammary tumor virus; RER, rough endoplasmic reticulum.

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trafficking pathways may ultimately be crucial to properly localize functioning glycoproteins to the cell surface; however, in most cases, the signaling molecules that regulate these processes have not been identified.

By examining the expression and sorting of cell surface mouse mammary tumor virus (MMTV) glycoproteins, we have uncovered evidence for a steroid-regulated intracellular trafficking pathway that is expressed in hepatic-derived tissue (9–13, 21). Dexamethasone, a synthetic glucocorticoid, regulates the posttranslational maturation and localization of a single glycosylated MMTV precursor polyprotein ($Pr74^{env}$) in M1.54 cells, a MMTV-infected rat hepatoma (HTC) cell line (13, 21). One-dimensional V8 protease peptide mapping revealed that identical MMTV glycosylated precursor polyproteins ($Pr74^{env}$) are initially synthesized in dexamethasone-induced and uninduced cells (21). In a manner similar to the biosynthesis of MMTV glycoproteins in other systems (54), each newly synthesized precursor is intercalated in the RER membrane and contains five endoglycosidase H–sensitive ("high mannose") oligosaccharides. Exposure to dexamethasone redirects the posttranslational expression of the glycosylated precursor resulting in the production of three new cell surface MMTV glycoproteins (gp78, gp70, and gp32) as well as an extracellular form of gp70. Both gp78 and gp70 represent different glycosylated forms of the same polyprotein in that both contain complex oligosaccharides but gp78 is sialyated to a greater extent. During transit through the cell, one or both of these polyproteins is proteolytically cleaved into an amino-terminal fragment (gp50) and the carboxy-terminal portion (gp32). The amino-terminal fragment, gp50, is constitutively externalized and is the predominant MMTV glycoprotein in uninduced cells (9, 10, 12, 13). In the presence of glucocorticoids, all of the viral glycoproteins are transported in association with the vesicle membranes (20); however, only gp78, gp70, and gp32 are stably integrated into the plasma membrane and acquire a lipid modification (11).

We have previously reported that the glucocorticoid-regulated trafficking of MMTV glycoproteins to the cell surface is a genetically distinct hormone response (10, 12) that requires receptor function and the de novo synthesis of cellular encoded gene products (11, 21, 27). To further define the reactions under glucocorticoid control, we used the well-characterized Na^+/K^+ ionophore, monensin (49), which has been shown to disrupt or slow down certain intracellular maturation and transport processes (18, 25, 33, 40, 58, 59). Monensin treatment simplified the overall pattern of MMTV protein maturation by inhibiting the proteolytic processing and externalization of the MMTV glycosylated polyprotein. These effects caused the stable cellular accumulation of a 70,000-M_r MMTV glycoprotein (gp70) and, therefore, allowed an analysis of the sorting of a single viral substrate that is destined for the cell surface only in the presence of glucocorticoids. By examining the kinetics of oligosaccharide processing of MMTV glycoproteins expressed in both wild-type hepatoma cells and a complement-selected sorting variant (10, 12, 27), we have localized a key glucocorticoid-regulated step in glycoprotein sorting to the Golgi region.

Materials and Methods

Materials

All media and sera used for tissue culture were purchased from the University of California, San Francisco Tissue Culture Facility. L-[^{35}S]Methionine (1,000 Ci/mmol), D-[2-^{3}H]mannose (16 ci/mmol), and [^{3}H]sodium borohydride (10 ci/mmol) were obtained from Amersham Corp. (Arlington Heights, IL) and EN^{3}HANCE from New England Nuclear (Boston, MA). Dexamethasone, monensin, and galactose oxidase were procured from Sigma Chemical Co. (St. Louis, MO), and Pansorbin and tunicamycin were acquired from Calbiochem-Behring Corp. (San Diego, CA [La Jolla]). Agarose derivatized with castor bean lectin (Ricin 120) was purchased from P-L Biochemicals (Milwaukee, WI) and Pharmacia Fine Chemicals (Piscataway, NJ), and x-ray film was obtained from Merry X-Ray Chemical Corp. (Burlingame, CA). Rhodamine isothiocyanate–conjugated goat anti-rabbit IgG was purchased from Cappel Laboratories (Malvern, PA). The anti-MMTV and preimmune sera (5) used in this study were generous gifts of L. J. T. Young and R. D. Cardiff (Department of Pathology, University of California, Davis). All other reagents were of the highest available purity.

Cells and Method of Culture

M1.54 is a cloned cell line isolated from MMTV-infected rat populations of cultured hepatoma cells (50); selection of its complement-resistant derivative, CR4, has been described previously (10, 12, 13). Both cell lines were propagated as monolayers in DME and radiolabeled with [^{35}S]methionine in methionine-free medium as described (21, 29). Where indicated, cell cultures were treated with 1 μM monensin and glucocorticoid-treated cells received 1 μM dexamethasone.

Pulse–Chase Experiments

Monolayer cultures were preincubated with 1 μM monensin for 1 h before treatment in the presence or absence of 1 μM dexamethasone. After 18 h, the cells were incubated for 40 min in methionine-free medium and then pulsed labeled with 50 μCi/ml [^{35}S]methionine for 10 min. After quickly rinsing the cells several times in PBS, the radiolabel was chased by incubating the cells in medium supplemented with 0.5% FCS and unlabeled methionine (0.3 mg/ml); appropriate concentrations of dexamethasone and monensin were present in the medium throughout both incubation periods. After 0, 30, 60, 90, 120, and 240 min of chase, cell monolayers were washed three times with ice-cold PBS, dislodged from the plates in 1 mM EDTA, 10 mM Tris-HCl, pH 7.5, in PBS, and centrifuged at 600 *g* for 10 min. The cell pellets were solubilized in 1% Triton X-100, 0.5% deoxycholate, 5 mM EDTA, 250 mM NaCl, 25 mM Tris-HCl, pH 7.5, and then applied to ricin affinity columns or digested with endoglycosidase H as described below. For the dexamethasone into chase experiment, uninduced cells were pulse labeled with 50 μCi/ml [^{35}S]methionine for 1 h, rinsed in PBS, and incubated with unlabeled methionine for 8 h in the presence or absence of 1 μM dexamethasone. The pulse-chase experiment using [2-^{3}H]mannose was accomplished as previously described (11).

Steady-State Incorporation of Radiolabeled Material and Recovery of Cellular and Secreted Fractions

Cells pretreated with or without 1 μM monensin for 1 h were incubated in the presence or absence of 1 μM dexamethasone for 16 h and subsequently radiolabeled in 30 μCi/ml [^{35}S]methionine for 4 h. Appropriate combinations of dexamethasone and monensin were present throughout the radiolabeling period. The secreted fractions were harvested by centrifugation of the culture medium at 2,000 *g* for 10 min; the resulting supernates were brought to 1% Triton X-100, 0.5% deoxycholate, and 5 mM EDTA just before immunoprecipitation. The cell monolayers were washed three times in ice cold PBS and incubated with MMTV antibodies as described below for the cell surface immunoprecipitations.

Galactose Oxidase-mediated Labeling of Cell Surface-associated Glycoproteins

24-h dexamethasone-induced and uninduced M1.54 cells were washed three times with PBS and incubated with 15 U galactose oxidase in 1 ml PBS/plate, for 5 min at 37°C (28). Cell monolayers were washed extensively with PBS, placed on ice for 2 min, and then gently overlayed with 1 ml PBS containing 1 mCi [^{3}H]sodium borohydride. Labeling was allowed to proceed for 5 min on ice, and the cells were then washed twice with PBS containing 10% horse serum to absorb the excess radiolabel, followed by two additional washes with PBS. Cells were then harvested, detergent solubilized, and radiolabeled glycoproteins were fractionated in SDS-polyacrylamide gels.

Cell Surface Immunoprecipitations

The expression of metabolically radiolabeled plasma membrane MMTV polypeptides were selectively examined by modifications of the method of Krangel et al. (32) as previously described (21). Intact cells were first incubated with 4 μl polyclonal anti-MMTV or preimmune sera in 1 ml PBS at 4°C for 15 min and then washed once with 10% horse serum in media, three times with PBS, and finally harvested. Cells were detergent solubilized as described above and the cell extracts incubated with 10 μl of 10% (wt/vol) Pansorbin (fixed *Staphylococcus aureus*) for 5 min to immunoadsorb the surface-associated antigen–antibody complexes. After centrifugation to recover the Pansorbin-pelletable (cell surface) material, more antibodies (8 μl of 1:10 dilution of serum) and Pansorbin (10 μl of 10% wt/vol) were added to the supernates to monitor expression of the intracellular MMTV proteins. Immunoprecipitations were continued as described below.

Immunoprecipitation of MMTV Glycoproteins, SDS Gel Electrophoresis, and Fluororadiography

Immunoprecipitations of cellular and secreted fractions were carried out with either anti-MMTV glycoprotein antibodies, total anti-MMTV antibodies, or with preimmune serum, using a highly sensitive low background Pansorbin assay previously described (26, 47). Final Pansorbin (*S. aureus*) pellets were solubilized in 25 μl SDS gel sample buffer (reference 34) and incubated with 5 μl of 0.5 M dithiothreitol (DTT) at 100°C for 2 min. After a 3-min Eppendorf centrifugation, the supernatant fractions were subjected to electrophoresis in SDS–polyacrylamide gels (42) containing 10% acrylamide. Proteins were subsequently fixed and stained by incubation overnight with 0.4% Coomassie Brilliant Blue then destained in 10% acetic acid for 5 h. Gels were impregnated with EN^{3}HANCE for 1 h, incubated in water for 1 h, dried under vacuum at 60°C, and analyzed by fluorography on Kodak RP-Royal X-Omat film at −70°C.

Endoglycosidase H Digestion

Sensitivity to the endoglycosidase H was assayed as previously described (19). Briefly, immunoprecipitated MMTV glycoproteins were released from antibody–*S. aureus* complexes in 30 μl 1% SDS at room temperature for 10 min, and the supernatant fractions added to 150 μl 100 mM sodium citrate buffer, pH 5.5, containing 80 mU/ml endoglycosidase H. Control incubations did not receive any enzyme. After 24 h at 37°C, the reactions were terminated by adding immunoprecipitation (solubilization) buffer containing MMTV antibody. The antibody–antigen complexes were then precipitated with *S. aureus* and fractionated in SDS–polyacrylamide gels.

Ricin–Agarose Column Chromatography

Ricin-agarose was poured to a bed volume of 0.5 ml and washed extensively with 0.1% Triton X-100, 150 mM NaCl, 10 mM Tris, pH 7.5 (ricin-binding buffer). Solubilized cell extracts were diluted to a final concentration of 0.1% Triton X-100 by the addition of 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, and applied to the ricin–agarose column, washed extensively in ricin-binding buffer; ricin-bound material was eluted with 3% galactose in ricin-binding buffer (19). Bound and nonbound fractions were immunoprecipitated and analyzed by SDS-PAGE.

Indirect Immunofluorescence

M1.54 and CR4 cells were transferred to polylysine-coated coverslips (4) and cultured overnight in the presence of 1 μM dexamethasone with and without 1 μM monensin, washed several times with PBS, and fixed by incubating with 3.7% formaldehyde in PBS for 20 min at room temperature. After washing extensively in PBS containing 10 mM glycine, the cells were incubated for 30 min on ice with 1 ml of an antibody solution containing 80 μl of a 1:10 dilution of anti-MMTV antibody, 100 μl of BSA solution (50 mg/ml), and 820 μl of PBS. The antibody solution was then aspirated off, cultures washed several times in PBS-glycine and then incubated with 500 μl PBS containing 120 μl of a 1:100 dilution of rhodamine-conjugated goat anti-rabbit IgG. After 30 min on ice, the coverslips were washed four times in PBS-glycine and then mounted on slides in 90% glycerol, 10% Tris-HCl (100 mM), pH 7.5 (55). Mounted slides were either examined immediately by fluorescence microscopy or stored at −20°C before visualization.

Transmission Electron Microscopy

Monolayer cultures of M1.54 and CR4 cells were treated with the indicated combinations of 1 μM dexamethasone and 1 μM monensin for 24 h and then fixed in 2% glutaraldehyde in 0.05 M cacodylate, pH 7.4, for 30 min. After rinsing in buffer, monolayers were then immersed in a solution of cacodylate containing 0.5% OsO_4, 0.8% $K_3Fe(CN)_6$ at 4°C for 15 min, followed by another buffer rinse, as described by McDonald (39). Cells were then placed in a 0.15% solution of tannic acid in buffer for 1 min and rinsed with buffer. The monolayers were then immersed in a 2% solution of aqueous uranyl acetate for 120 min. Cells were infiltrated after dehydration and embedded in Medcast-Araldite 6005 (Pelco Corp., Tustin, CA) as previously described (2). 0.8-μm sections were placed on copper grids, and stained with uranyl acetate and lead citrate before analysis using a Philips 300 transmission electron microscope.

Assay of Membrane-associated Galactosyl Transferase Activity

Cells were treated in the presence or absence of 1 μM dexamethasone for 24 h, harvested, incubated in a hypotonic swelling buffer (20 mM KCl, 20 mM Tris-HCl, pH 7.2) at 4°C for 10 min, and broken by homogenization in a Dounce glass homogenizer (Kontes Glass Co., Vineland, NJ). Postnuclear supernates were isolated by centrifugation at 2,000 *g* for 10 min and the microsomal membranes pelleted after centrifugation at 32,000 rpm for 90 min at 4°C in a Ti40 rotor. The microsomal membranes were resuspended in 1 ml of 0.25 M sucrose, 1 mM EDTA, 10 mM Tris-HCl, pH 7.0, aspirated through a 23-gauge needle 10 times, and fractionated on a 65–15% sucrose gradient by centrifugation for 16 h at 25,000 rpm in an SW28 rotor; 2.5-ml fractions were collected, diluted to 14 ml in the hypotonic swelling buffer, and repelleted by centrifugation at 32,000 rpm in a Ti40 rotor for 1 h at 4°C. The resulting pellets were resuspended in 0.25 M sucrose, 1 mM EDTA, 10 mM Tris-HCl, pH 7.0, by aspiration through a 23-gauge needle and assayed for galactosyl transferase activity using UDP-[^{3}H]galactose and ovomucoid as an acceptor glycoprotein (51). The peak galactosyl transferase activity migrated in all gradients between 0.7 and 0.95 M sucrose.

Results

Monensin-mediated Expression of a Single MMTV Glycosylated Polyprotein and Its Selective Localization to the Cell Surface in a Glucocorticoid-dependent Manner

Dexamethasone-induced and uninduced M1.54 cells were incubated in the absence or presence of monensin (1 μM) before and throughout a 4-h radiolabeling period with [^{35}S]-methionine. By adding anti-MMTV antibodies to intact cells, we were able to design immunoprecipitations that directly distinguish [^{35}S]methionine-labeled cell surface-associated polypeptides from intracellular MMTV polypeptides expressed in the same sample of cells. Extracellular viral glycoproteins were analyzed by immunoprecipitation of the conditioned culture media. Consistent with our previous results (10, 12, 21), several new cell surface–associated (gp78, gp70, and gp32; Fig. 1, lane *E* vs. *G*) and extracellular (gp70) MMTV glycoproteins (lane *I* vs. *K*) and two new intracellular viral phosphoproteins (p35 and p24; lane *A* vs. *C*) were uniquely expressed in dexamethasone-treated M1.54 cells. Exposure to monensin selectively altered the pattern of expressed MMTV proteins. The predominant intracellular viral glycoprotein detected in both hormone-induced (lane *D*) and uninduced (lane *B*) cells treated with monensin was a stable form of a 70,000-M_r polyprotein (designated gp70). The Pr74 species shown in this gel represents the phosphorylated precursor polyprotein which is encoded by the viral gag gene and is selectively immunoprecipitated with anti-phosphoprotein antibodies (12, 29). Even though intracellular gp70 was clearly observed in the presence or absence of hormone (lanes *B* and *D*), its translocation to the cell surface was observed only after exposure to dexamethasone (lane *H* vs. *F*).

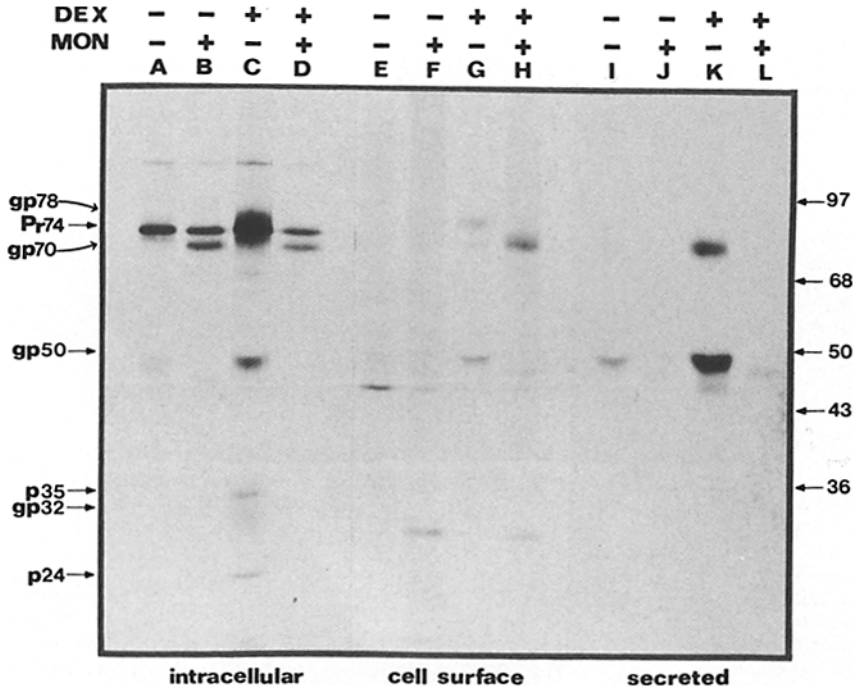

Figure 1. Glucocorticoid-regulated expression of intracellular, cell surface, and extracellular MMTV proteins. Dexamethasone-induced (lanes *C, D, G, H, K,* and *L*) and uninduced (lanes *A, B, E, F, I,* and *J*) M1.54 cells were treated in the presence (lanes *B, D, F, H, J,* and *L*) or absence (lanes *A, C, E, G, I,* and *K*) of monensin, radiolabeled with [^{35}S]methionine, and the expression of intracellular (lanes *A–D*), cell surface (*E–H*), and secreted (*I–L*) viral proteins were analyzed by immunoprecipitation, as described in the text. After fractionation in SDS–polyacrylamide gels, radioactive protein bands were visualized by fluorographic display. The molecular weight standards are phosphorylase b (97,000 M_r), BSA (68,000 M_r), bovine gamma globulin (50,000 M_r), ovalbumin (43,000 M_r), glyceraldehyde phosphate dehydrogenase (36,000 M_r), and trypsin inhibitor (20,000 M_r).

Densitometric analysis of the autoradiograph (Fig. 1, lane *G* vs. *H*) revealed that in hormone-induced cells, ionophore treatment caused an ~20-fold accumulation of gp70 at the cell surface. This striking accumulation may have resulted in part from its failure to be exfoliated into the extracellular environment (Fig. 1, lane *K* vs. *L*). Consistent with this notion, pulse–chase analysis of dexamethasone-induced cells revealed that gp70 reaches the cell surface in the presence or absence of ionophore, but its subsequent release into the culture media was selectively blocked in monensin-treated cells (data not shown). It is important to point out that hormone-induced M1.54 cells not exposed to monensin externalize a 70,000-M_r species (gp70), although both a 78,000- and 70,000-M_r species (gp78 and gp70) are detected at the cell surface (Fig. 1 and reference 21). In a manner similar to the shedding effect normally observed in MMTV-expressing cell lines (54), externalized gp70 may be derived from one of the plasma membrane–associated polyproteins.

The ionophore-mediated accumulation of cell surface gp70 in hormone-induced cells was further confirmed by an ~20-fold increase in [^{125}I]gp70 labeled by lactoperoxidase iodination of intact cells and by an increased sensitivity to anti-MMTV antibody-directed complement-mediated cytolysis (data not shown). Thus, in the presence of monensin, gp70 accumulated at the cell surface only in glucocorticoid-treated cells, whereas, in uninduced cells, gp70 remained in an unidentified intracellular fraction. Regardless of the precise mechanism, exposure to monensin provided the ability to follow the intracellular sorting of a single well-defined

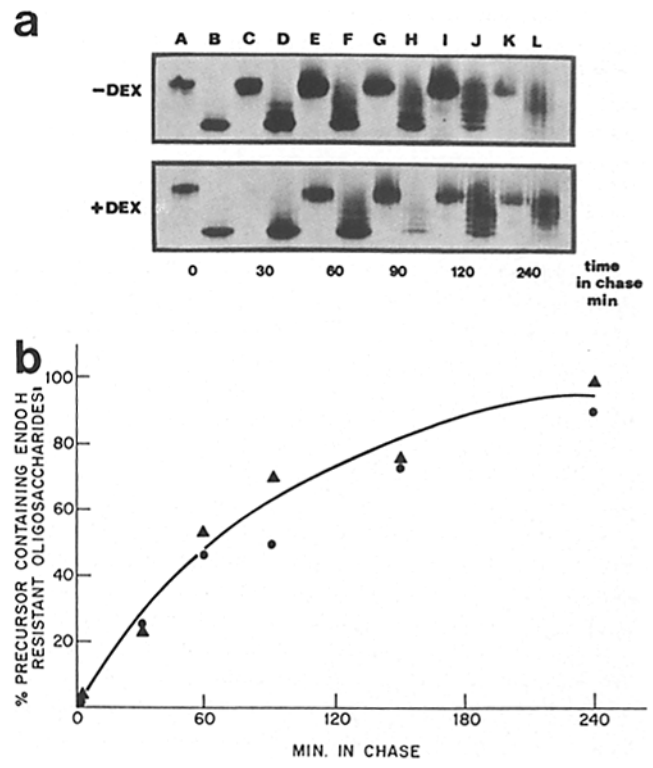

Figure 2. Endoglycosidase H digestion of gp70 expressed in glucocorticoid-induced and uninduced M1.54 cells. (*a*) Dexamethasone-treated (*lower gel*) and untreated (*upper gel*) M1.54 cells were pulsed labeled with [^{35}S]methionine for 5 min and incubated with excess unlabeled methionine for the indicated chase periods. Cells were solubilized in detergents and duplicate aliquots immunoprecipitated with anti-MMTV glycoprotein antibodies. The viral glycoproteins were released from Pansorbin in 1% SDS, digested with (lanes *B, D, F, H, J,* and *L*) and without (lanes *A, C, E, G, I,* and *K*) endoglycosidase H, and fractionated in SDS–polyacrylamide gels as described in the text. (*b*) The percent of gp70 containing endoglycosidase H–resistant oligosaccharides (Endo H–resistant gp70/total gp70) through the pulse–chase time course (*b*) was quantitated by scanning the fluorogram of Endo H–digested samples with a soft laser densitometer. ●, −DEX; ▲, +DEX.

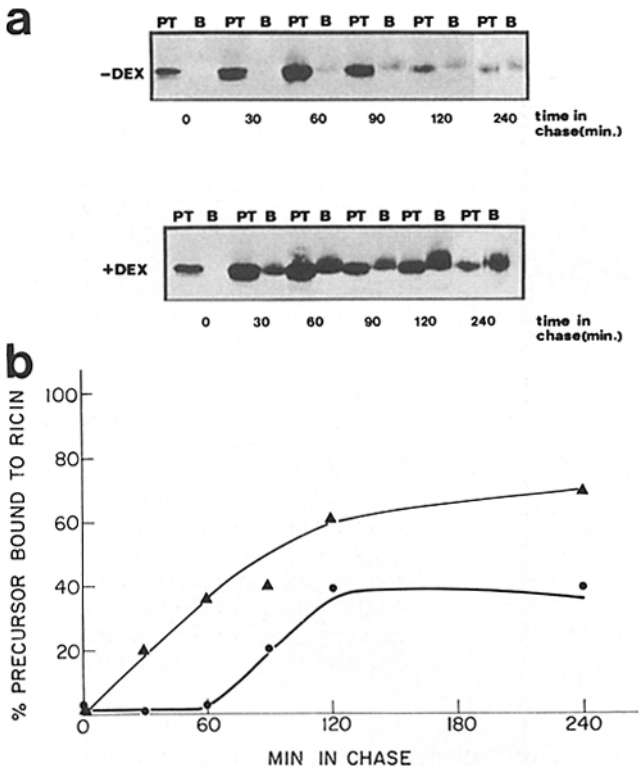

Figure 3. Ricin affinity chromatography of gp70 expressed in glucocorticoid-induced and uninduced cells. (*a*) Through the same pulse-chase time course as described in Fig. 2, detergent-solubilized extracts from hormone-induced (*lower gel*) and uninduced (*upper gel*) M1.54 cells were applied to ricin affinity columns and galactose-containing glycoproteins eluted with 3% galactose as described in the text. Pass-through (*PT*) and ricin-binding (*B*) material were immunoprecipitated with anti-MMTV glycoproteins and fractionated in SDS–polyacrylamide gels. (*b*) The percent gp70 bound to ricin (B/[PT + B] × 100%) through the pulse–chase time course (*b*) was quantitated by soft laser densitometry of the fluorograms. ●, − DEX; ▲, +DEX.

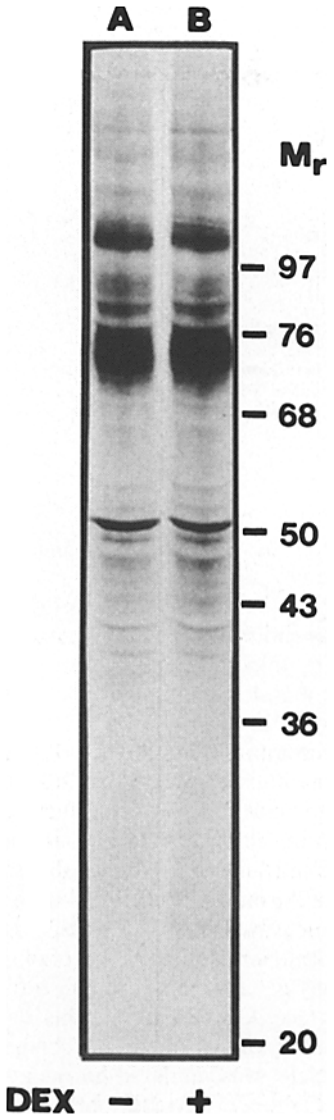

Figure 4. Analysis of galactose-containing cell surface glycoproteins in glucocorticoid-induced and uninduced M1.54 cells. M1.54 cells were treated in the presence (lane *B*) or absence (lane *A*) of 1 μM dexamethasone for 24 h and the galactose residues of cell surface glycoproteins labeled in intact cells by the galactose oxidase and NaB[^{3}H]$_4$ procedure described in the text. Radiolabeled cell extracts were fractionated in SDS–polyacrylamide gels and galactose-containing species visualized by fluorography. The molecular weight standards are the same as Fig. 1 with the addition of conalbumin (76,000 M_r).

substrate, gp70, which can be used to explore the hormone-inducible process that mediates the localization of cell surface glycoproteins.

Analysis of MMTV Glycoprotein Transport by Oligosaccharide-processing Kinetics in Glucocorticoid-induced and Uninduced Cells

To define the approximate intracellular site where glucocorticoids affect glycoprotein trafficking, the oligosaccharide processing state of gp70 was assayed through a pulse–chase time course. Resistance to endoglycosidase H was used to monitor the transport of gp70 from the RER to the medial Golgi (8, 24, 31, 48). Dexamethasone-induced M1.54 cells were treated with monensin for 16 h and radiolabeled for 10 min with [^{35}S]methionine followed by additional incubation in the presence of excess unlabeled methionine. At the indicated times, radiolabeled MMTV glycoproteins were immunoprecipitated and incubated in the presence or absence of endoglycosidase H. As shown in Fig. 2 (fluorographs of immunoprecipitated gp70), in both dexamethasone-treated and -untreated cells, virtually all of gp70 acquired at least one endoglycosidase H–resistant oligosaccharide side chain with a 65-min half time (Fig. 2 *b*). Thus, hormone treatment did not affect the apparent rate by which gp70 is transported from the RER to the medial Golgi region.

Transport of gp70 between the medial and *trans* cisternae of the Golgi was studied through the same chase points, by examining the rate and extent of galactose attachment (6, 8, 48). Detergent-solubilized extracts from monensin-treated cells were applied to ricin-derivatized agarose affinity columns and the galactosylated glycoproteins that bound ricin eluted with 3% galactose. Both the nongalactosylated material that passed through the column (*PT*) and bound (*B*) fractions were assayed for gp70 by immunoprecipitation with anti-MMTV glycoprotein antibodies (Fig. 3 *a*). The galactosylation of gp70 was strikingly altered in dexamethasone-induced cells. Densitometric quantitation of the proportion of gp70 that bound ricin revealed that in uninduced cells gp70 inefficiently acquired galactosyl residues after an initial 1-h lag, whereas in hormone-treated cells, >70% of gp70 acquired this sugar modification with no apparent time lag (Fig. 3 *b*). Thus, the efficient galactosylation of gp70 can be strongly correlated with its entry into a glucocorticoid-

Table I. Expression of Galactosyl Transferase Activity by Glucocorticoid-induced and Uninduced Wild-type and Variant Cell Lines

Cell line	Dexamethasone	Galactosyl transferase specific activity
		*nmol/30 min per mg**
M1.54	−	4.02
M1.54	+	2.85
CR4	−	4.99
CR4	+	3.32

* Postnuclear supernates from dexamethasone-treated and untreated cells were centrifuged at 100,000 *g* for 90 min and the resulting microsomal pellets resuspended and applied to a 65–15% sucrose gradient. Galactosyl transferase activity was monitored by the formation of [^{3}H]galactose-ovomucoid from UDP-[^{3}H]galactose in each gradient fraction and the specific activity of the peak fractions calculated.

regulated transport route within the Golgi complex and its subsequent localization to the cell surface.

Most likely, attachment of galactose residues occurs distal to, or at, the actual hormone-induced regulatory step in MMTV glycoprotein transport. Importantly, however, several lines of evidence suggest that dexamethasone does not affect general competence for the production of galactosylated glycoproteins. The expression of total galactosylated glycoproteins at the cell surface was assayed by incubation of intact cells with galactose oxidase followed by reduction of the oxidized galactose residues with NaB[^{3}H]$_4$. As shown in Fig. 4, the identical array of cell surface galactose-labeled glycoproteins were detected in hormone-induced and uninduced cells not exposed to monensin. Thus, glucocorticoids appear to regulate the trafficking of a restricted subset of glycoprotein substrates since many glycoproteins are constitutively localized to the cell surface. Consistent with this result, the specific activity of galactosyl transferase in membrane fractions enriched in Golgi vesicles was similar in hormone-induced and uninduced cells (Table I). Furthermore, dexamethasone treatment had no effect on the extent and rate of [^{3}H]galactose attachment to total cellular glycoproteins (data not shown). These experiments were accomplished in the absence of monensin although ionophore treatment per se did not significantly affect galactosyl transferase activity and the synthesis of galactosylated cellular and cell surface glycoproteins, nor did it affect the efficient galactosylation of gp70 in the presence of hormone (data not shown).

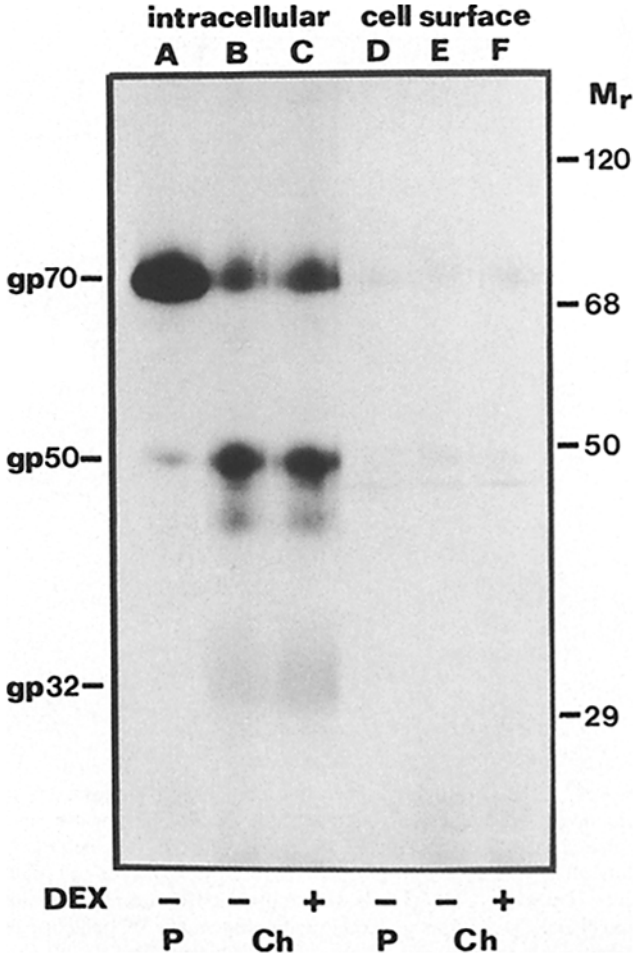

Figure 5. Analysis of [^{35}S]methionine-labeled intracellular and cell surface MMTV glycoproteins pulse labeled in uninduced cells and chased in the presence or absence of dexamethasone. Uninduced M1.54 cells were radiolabeled with [^{35}S]methionine for 1 h (lanes *A* and *D*) and incubated with excess unlabeled methionine in the presence (lanes *C* and *F*) or absence (lanes *B* and *E*) of 1 μM dexamethasone for 8 h. Cell surface (lanes *D–F*) and intracellular (lanes *A–C*) MMTV glycoproteins were analyzed by immunoprecipitation as described in the text. Immunoprecipitated material were fractionated in SDS-polyacrylamide gels and radiolabeled proteins were visualized by fluorographic display. The molecular weight standards are described in Fig. 1 with the addition of β-galactosidase (120,000 M_r).

Trafficking of gp70 Proceeds into a "Dead End" Pathway in Uninduced Cells

Conceivably, in uninduced cells, gp70 may be sequestered in a cell compartment which prevents its subsequent compartmentalization to the cell surface, or it may be transported up to and no further than the glucocorticoid-regulated reaction. To test these possibilities, uninduced cells not treated with monensin were pulse labeled with [^{35}S]methionine for 1 h and incubated with excess unlabeled methionine for 8 h either in the presence or absence of dexamethasone. An 8-h chase period was used since 4-h exposure to hormone is required for expression of gp70 at the cell surface (21). The presence of intracellular and cell surface MMTV glycoproteins were analyzed by immunoprecipitation. As shown in Fig. 5, gp70 failed to be detected at the cell surface both with or without the addition of dexamethasone during the chase incubation (lanes *F* and *E*) and remained in an intracellular fraction (lanes *C* and *B*). The reduced levels of gp70 detected after an 8-h chase relative to the pulse point most likely reflect proteolytic processing into gp50 and gp32. These results suggest that in uninduced cells, newly synthesized gp70 is sequestered in an intracellular compartment that does not allow its subsequent transport to the cell surface even after hormonal exposure.

Analysis of Cellular Morphology by Electron Microscopy

To examine potential effects of dexamethasone treatment on subcellular morphology, cross sections of hormone-induced

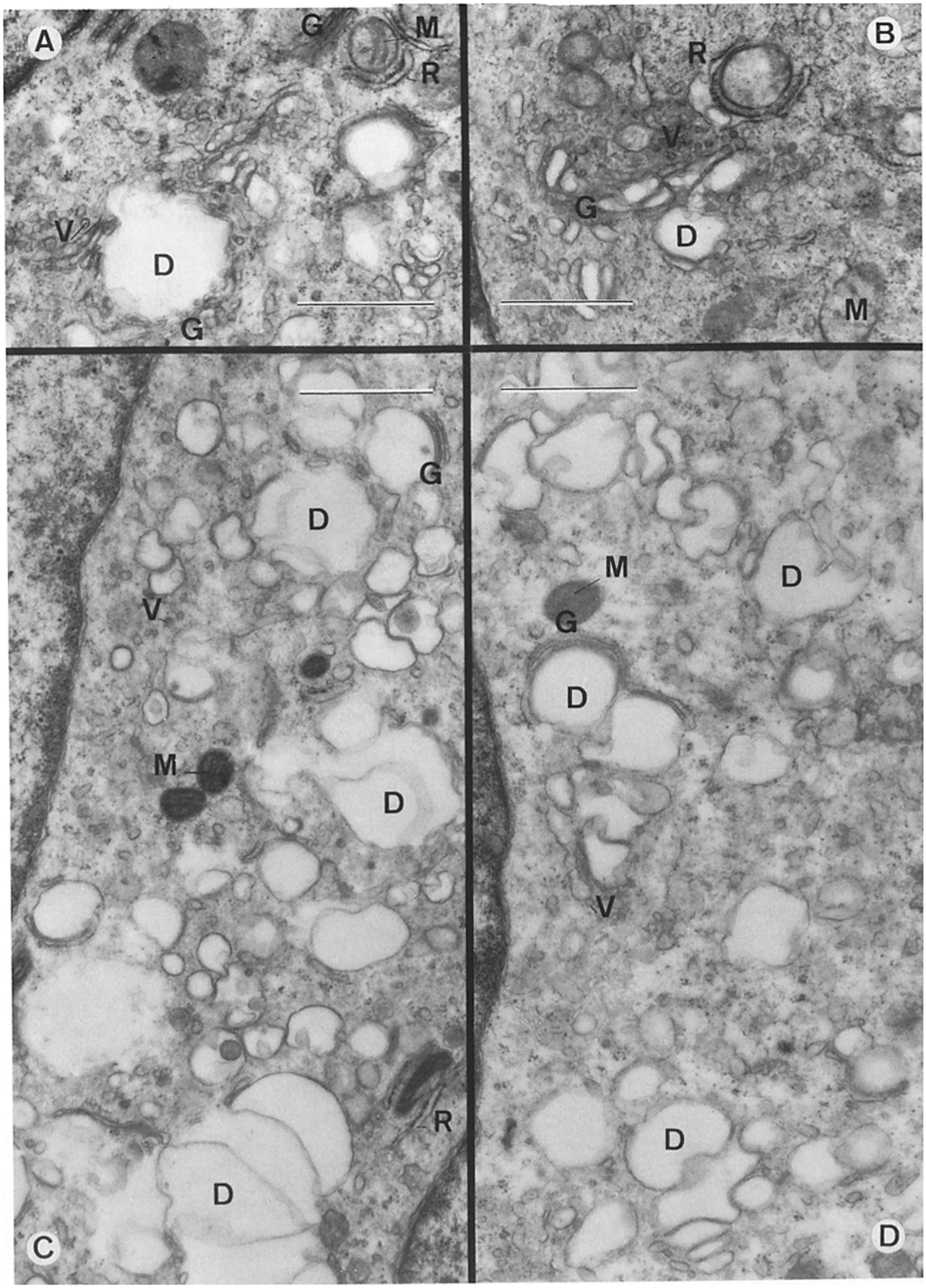

Figure 6. Electron microscopic analysis of glucocorticoid-induced and uninduced M1.54 cells. Monolayer cultures of M1.54 cells were treated with the following combinations of 1 μM monensin and 1 μM dexamethasone for 24 h: (*A*) no additions; (*B*) dexamethasone; (*C*) monensin; and (*D*) dexamethasone and monensin. Monolayers were fixed, poststained with osmium ferricyanide, and analyzed by transmission electron microscopy as described in the text. *G*, Golgi cisternae; *D*, dilated vacuole; *V*, Golgi-associated vesicles; *M*, mitochondria; and *R*, rough endoplasmic reticulum. Bars, 1 μm.

and uninduced monolayer cultures of M1.54 cells, treated in the presence or absence of monensin, were analyzed by transmission electron microscopy. Perinuclear areas shown in Fig. 6 revealed that the stacking and vesicularization of Golgi-like vesicles were similar in the presence or absence of hormone (Fig. 6, *B* vs. *A* and *D* vs. *C*). In the absence of ionophore, the Golgi region typically observed with (Fig. 6 *B*) and without (Fig. 6 *A*) hormone treatment consisted of cisternae surrounding a dilated vacuole; occasional flattened cisternae without an accompanying vacuole were also observed. Thus, under conditions in which dexamethasone treatment affects the intracellular sorting of MMTV glycoproteins, hormone treatment had no detectable effects on the Golgi morphology. As expected (59), monensin increased the relative number and sizes of vesicular structures dispersed within the perinuclear area (Fig. 6, *C* and *D* vs. *A* and *B*). In general, most of the Golgi elements are replaced by dilated vacuoles with some normal-appearing Golgi elements. Importantly, dexamethasone treatment had no additional affects on general vesicular structure.

Localization and Galactosylation of MMTV Glycoproteins in the Sorting Variant CR4

In a previously characterized sorting variant, CR4, the overall pattern of MMTV glycoprotein maturation in the presence of dexamethasone was strikingly similar to uninduced wild-type M1.54 cells (12). Visualization by electron microscopy revealed that the overall subcellular morphology of variant CR4 is virtually identical to wild-type M1.54 cells after dexamethasone and/or monensin treatment (data not shown). Indirect immunofluorescence demonstrated that dexamethasone-treated wild-type M1.54 cells displayed a clearly visible capping of anti-MMTV antibody-directed fluorescence in the presence or absence of monensin indicative of cell surface–associated viral antigens (Fig. 7, *b* and *d*). In contrast, hormone-induced CR4 failed to localize MMTV glycoproteins to the cell surface either in the presence or absence of monensin (Fig. 7, *f* and *h*). Under these conditions, immunofluorescent labeling of intracellular membrane–associated MMTV glycoproteins is clearly visible in detergent permeabilized cells (data not shown).

The kinetics of gp70 galactosylation in hormone-induced CR4 treated with monensin was monitored by the ricin-binding assay through an identical pulse–chase time course as described for Fig. 3. As shown in Fig. 8, hormone-induced CR4 competently produced gp70 and, like uninduced wild-type cells (Fig. 3), this glycoprotein remained in an intracellular fraction and ungalactosylated. The galactosylation of MMTV glycoproteins was also monitored in wild-type M1.54 and variant CR4 cells not exposed to ionophore. Hormone-induced cells were pulse labeled with [2-^{3}H]mannose for 10 min and incubated with excess unlabeled mannose for 90 min. Analysis of galactosylation by ricin binding revealed that after 90 min, ~60% of MMTV glycoproteins bind to ricin and are eluted with 3% galactose (Fig. 9, lane *G* vs. *E*); the three major ricin-binding species are gp78, gp70, and gp50 (Fig. 9, lane *G*). In contrast, most MMTV glycoproteins remain ungalactosylated in CR4 (Fig. 9, lane *O* vs. *M*). As expected, the RER form of the viral-glycosylated polyprotein synthesized in wild-type (lane *A*) and variant (lane *I*) cells during the 10-min pulse failed to bind ricin since galactoslyation is a Golgi-mediated reaction.

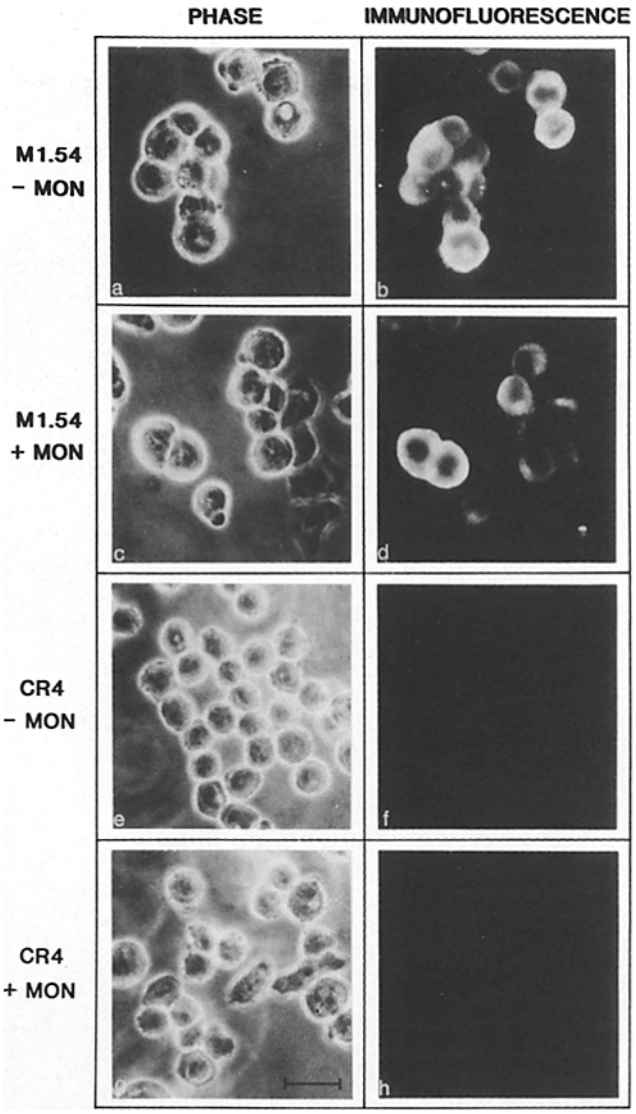

Figure 7. Immunofluorescence analysis of cell surface MMTV glycoproteins. M1.54 and CR4 cells were plated onto polylysine-coated coverslips, incubated in the presence (*c*, *d*, *g*, and *h*) or absence (*a*, *b*, *e*, and *f*) of 1 μM monensin and treated with 1 μM dexamethasone for 16 h. Cells were fixed in formaldehyde and incubated sequentially with anti-MMTV antibodies and rhodamine-conjugated goat anti–rabbit IgG as described in the text. Populations of cells were examined by both phase (*a*, *c*, *e*, and *g*) and fluorescence (*b*, *d*, *f*, and *h*) microscopy. Bar, 30 μm.

The level of membrane-associated galactosyl transferase activity (Table I) and bulk expression of galactosylated glycoproteins (data not shown) were unaffected in CR4. Importantly, in a manner similar to hormone-induced M1.54 cells, dexamethasone-treated CR4 produces MMTV glycoproteins with endoglycosidase H–resistant oligosaccharides (12) demonstrating that the RER-to-Golgi transport of MMTV glycoproteins in CR4 was not defective. Taken together, our hormonal and genetic results suggest that MMTV glycoproteins enter the early Golgi cisternae by a constitutive route and are routed by a glucocorticoid-regulated step in the Golgi before or at the site of galactose attachment.

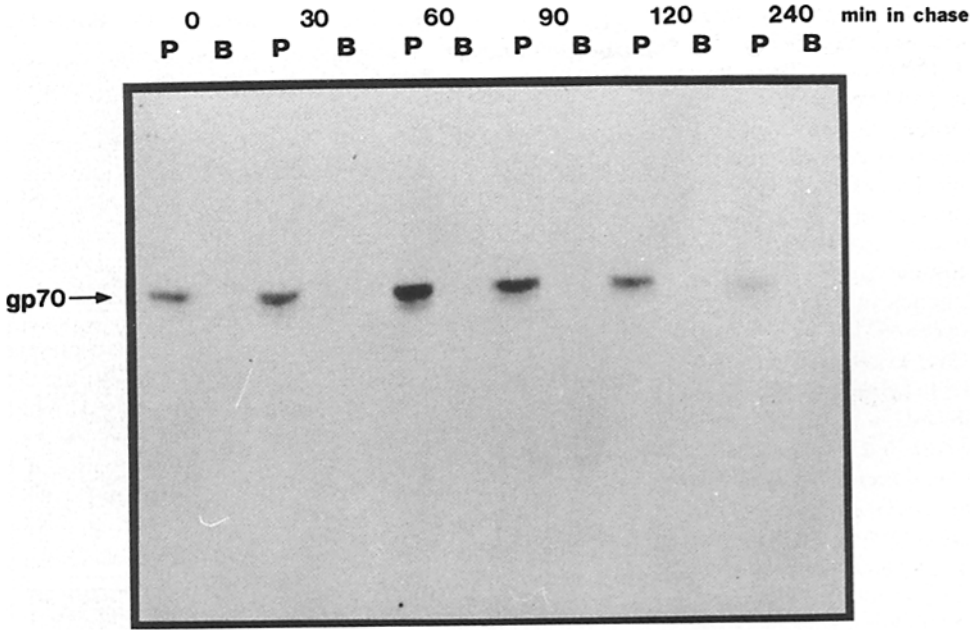

Figure 8. Ricin affinity chromatography of gp70 expressed in glucocorticoid-induced, monensin-treated CR4 cells. Dexamethasone-treated CR4 cells were pulsed labeled with [^{35}S]methionine for 5 min and incubated with excess unlabeled methionine for the indicated chase periods. Detergent-solubilized extracts were applied to ricin affinity columns and galactose-containing glycoproteins eluted with 3% galactose as described in the text. Pass-through (*P*) and ricin-binding (*B*) material were immunoprecipitated with anti-MMTV glycoproteins and fractionated in SDS–polyacrylamide gels.

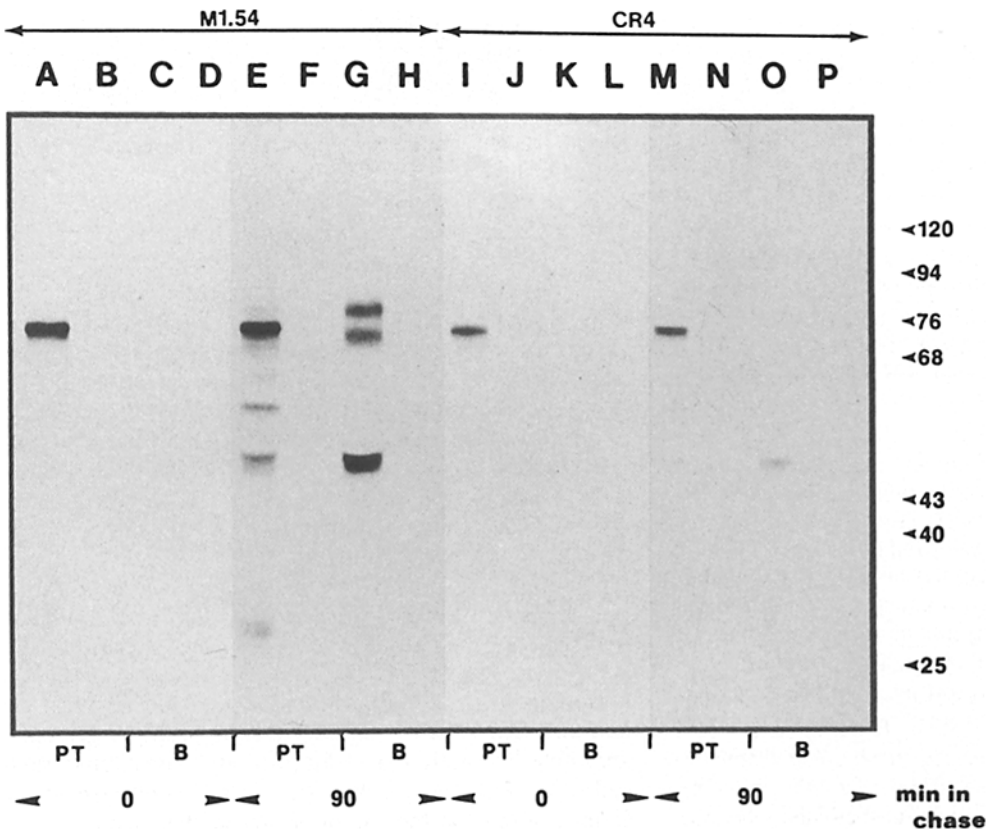

Figure 9. Ricin affinity chromatography of MMTV glycoproteins expressed in dexamethasone-induced wild-type cells and a sorting variant in the absence of monensin. Cultures of M1.54 and CR4 cells were exposed to 1 μM dexamethasone for 24 h and then pulse labeled with 100 μCi [2-^{3}H]mannose for 20 min followed by further incubation in the presence of excess unlabeled mannose for 90 min; dexamethasone was present throughout the experiment. Cellular fractions were harvested after the pulse (lanes *A–D* and *I–L*) and 90-min chase (lanes *E–H* and *M–P*). Detergent-solubilized extracts were applied to ricin affinity columns and galactose-containing glycoproteins eluted with 3% galactose as described in the text. Pass-through (*PT*) and ricin-binding (*B*) material were immunoprecipitated with anti-MMTV glycoprotein antibodies (lanes *A*, *C*, *E*, *G*, *I*, *K*, *M*, and *O*) or preimmune serum (lanes *B*, *D*, *F*, *H*, *J*, *L*, *N*, and *P*) and fractionated in SDS–polyacrylamide gels. The molecular weight standards are the same as described in Figs. 1, 4, and 5 with the addition of phosphorylase a (94,000 M_r), aldolase (40,000 M_r), and chymotrypsinogen (25,000 M_r).

Discussion

By monitoring the kinetics and efficiency of MMTV glycoprotein oligosaccharide-processing in wild-type and variant hepatoma cells, we have uncovered a glucocorticoid-regulated step in the trafficking of membrane glycoproteins that occurs within the medial or *trans*-Golgi cisternae. For some of our studies, we exploited the Na^+/K^+ ionophore monensin, which simplified the normally complex maturation pattern of MMTV glycoproteins. Similar to other reported membrane proteins (25, 58, 60), monensin significantly reduced the proteolytic processing of the MMTV glycosylated polyproteins and additionally prevented their externalization. Under these conditions, a stable cellular 70,000-M_r viral glycoprotein (gp70) accumulated in both hormone-induced and uninduced wild-type cells but was localized to the cell surface only after exposure to dexamethasone. Thus, despite the known global effects on cellular morphology and protein transport (18, 40, 58), treatment with monensin allowed a selective analysis of the glucocorticoid-regulated trafficking of relatively high concentrations of a single plasma membrane–directed substrate. Dexamethasone treatment did not simply overcome a monensin-mediated block in protein trafficking within wild-type M1.54 cells, since in the presence or absence of ionophore MMTV glycoproteins are transported to the cell surface only after hormone induction. Furthermore, ionophore treatment did not alter the phenotype of a sorting variant, CR4, which fails to galactosylate and transport MMTV glycoproteins to the cell surface in the presence of dexamethasone.

Other investigators have suggested that the *trans*-Golgi network, which is morphologically contiguous with the *trans*-cisternae of the Golgi region, provides a regulatory site for the differential trafficking of secreted and lysosomal proteins (17). In nonpolarized cells, certain membrane-associated proteins appear to be constitutively transported from this network (17). Recently, an epithelial organelle, the vacuolar apical compartment, was described in polarized MDCK cells which selectively stored apically destined proteins (61). In addition, for some cell surface (and secreted) proteins, a rate-limiting step in intracellular transport has been observed in the RER-to-Golgi translocation process (14, 35, 36, 63). The novel feature of our results is the detection of a glucocorticoid-induced regulatory site within the Golgi complex after the acquisition of endo-β-*N*-acetylglucosaminidase H–resistant oligosaccharides but before or at the attachment site of galactose that alters the intracellular sorting of membrane-associated glycoproteins. Glucocorticoids do not affect the bulk localization of cell surface–associated glycoproteins containing galactose or the expression of galactosyl transferase activity (Fig. 4 and Table I) suggesting that a unique subset of glycoproteins is apparently transported through the glucocorticoid-regulated pathway while most membrane species are routed through a nonregulated or constitutive pathway. The precise mechanism is unknown and glucocorticoids may regulate any one of many types of cellular trafficking reactions that include an attenuation of an actual branchpoint in glycoprotein sorting, a selective stimulation in the vesicular export rate from certain Golgi cisternae, or a processing activity that acts on MMTV glycoproteins.

In hormone-induced cells, MMTV glycoproteins are efficiently transported to the cell surface, while in uninduced cells these same substrates enter constitutively into a dead end compartment from which they cannot be transported to the cell surface even after hormone treatment. Part of the specificity of this phenomenon most likely results from the actions of vesicle- or organelle-associated "sorting carriers," analogous to mannose-6-phosphate receptors (8, 57) and β_2-microglobulin (32, 44) that bind and help segregate or retain specific glycoprotein substrates into specific intracellular compartments or through certain sorting routes. Given current concepts in steroid hormone action (1, 65), glucocorticoids might directly induce specific sorting receptors that act within the Golgi complex to actively direct glycoproteins into an intracellular route, which delivers them to the cell surface, or by regulating the synthesis of blocking activities that selectively prevent certain glycoproteins from entering the constitutive route. Perhaps processing activities that modify MMTV glycoproteins such that they acquire a specific sorting signal may also be under hormonal control. In this regard, we have previously reported that the MMTV glycosylated precursors synthesized in hormone-induced and uninduced cells are structurally identical (21) suggesting that the steroid-mediated modification of gp70 is an unlikely event. Whatever the mechanism, the hormone-regulated sorting of viral glycoproteins required de novo RNA synthesis (21, 26) indicating the selective involvement of newly synthesized glucocorticoid-inducible components. Furthermore, these regulated components appear mutable since MMTV glycoproteins failed to be translocated to the cell surface and were inefficiently galactosylated into dexamethasone-treated variant CR4 cells (Figs. 7–9).

This study has shown that glucocorticoids regulate the intracellular sorting of MMTV glycoproteins destined for the cell surface; the hormone-inducible event does not affect RER-to-Golgi transport but facilitates the trafficking of specific glycoproteins through the Golgi region. The modulation of even a single posttranslational event could strongly influence the functional expression of many hepatoma cell glycoprotein products. In principle, such a pathway may be used to acutely regulate the trafficking of multiple, perhaps functionally related, sets of proteins during the development and function of normal liver. In fact, a series of fusion experiments have revealed that normal rat hepatocytes functionally complement the defect in variant CR4 confirming that normal liver expresses components of the glucocorticoid-regulated trafficking pathway (26). Through further characterization of MMTV glycoprotein trafficking in both wild-type and variant cell lines, it should be possible to define the precise posttranslational reactions that are under control of glucocorticoid hormones as well as identify the other cellular encoded glycoprotein substrates of this pathway.

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